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MASTER

Purification of Phospholamban, a 22,000 Dalton Protein from Cardiac Sarcoplasmic Reticulum that is Specifically Phosphorylated by Cyclic AMP - Dependent Protein Kinase*

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REA

DISCLAIMER

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Running Title: Purification of Phospholamban

SUMMARY

Very low concentrations of the detergent, deoxycholate (DOC), have been used to isolate two functionally interesting proteins from canine cardiac sarcoplasmic reticulum. These two proteins are phospholamban, a 22,000 dalton protein, specifically phosphorylated by adenosine 3':5'-monophosphate (cyclic AMP) - dependent protein kinase, and the Ca^{2+} + Mg^{2+}-ATPase, the major protein of the sarcoplasmic reticulum, responsible for the active transport of calcium. When phosphorylated, phospholamban is not solubilized by DOC. However, unphosphorylated, phospholamban is soluble in a very low concentration of DOC. This finding is used in the purification of phospholamban. The 22,000 dalton protein is first solubilized in a very low concentration of DOC and then subjected to column chromatography. After molecular weight sieving on a Sephadex G-75 column, the 22,000 dalton protein appears as a purified protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The purified protein is specifically phosphorylated by cyclic AMP-dependent protein kinase, 0.15 moles phosphate/mole protein. Phospholipids are still bound to the isolated 22,000 dalton protein in the ratio of 5 moles phospholipid to 1 mole protein.

The Ca^{2+} + Mg^{2+}-ATPase is purified by first solubilizing all the extrinsic proteins with a low concentration of DOC. An increasing amount of DOC is then added to yield the purified Ca^{2+} + Mg^{2+}-ATPase. This protein is at least 95% pure as determined by SDS-polyacrylamide gels. Adding additional DOC to the purified enzyme enhances the enzyme's ability to hydrolyze ATP to approximately 2.5 μmoles Pi/mg/min. The isolation of the 22,000 dalton protein and the Ca^{2+} + Mg^{2+}-ATPase will aid in understanding how these two proteins function and if they specifically interact with one another.

INTRODUCTION

Purification of a protein is a major step toward understanding the function of the protein. In order to purify most membrane bound proteins, it is first necessary to separate the protein from the lipid membrane. Detergents have been generally used to solubilize proteins (1,2). This communication reports on the purification of two proteins from canine cardiac sarcoplasmic reticulum. Very low concentrations of detergent were used to selectively solubilize these proteins, greatly aiding in their purification.

In canine cardiac sarcoplasmic reticulum, cyclic AMP-dependent protein kinase[1] enhances calcium transport into the sarcoplasmic reticulum (3). Cyclic AMP-dependent protein kinase also specifically phosphorylates two proteins, one with a molecular weight of 6,000 and one with a molecular weight of 22,000 (4,5). The 22,000 dalton protein, named phospholamban, has been identified and studied by others (6-10). It exhibits characteristics of phosphoester bonding (6). The phosphorylation of the 22,000 dalton protein parallels the enhancement of calcium transport into the sarcoplasmic reticulum, when cyclic AMP and protein kinase are present (7). The phosphorylation and dephosphorylation of the 22,000 dalton protein is believed to be involved in the regulation of calcium transport into cardiac sarcoplasmic reticulum.

The Ca^{2+} + Mg^{2+}-ATPase, a 100,000 dalton protein, is the integral protein of both cardiac and skeletal sarcoplasmic reticulum. It actively transports calcium into the sarcoplasmic reticulum. The ATPase from

[1]The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate; protein kinase, adenosine 3':5'-monophosphate - dependent protein kinase; EGTA, ethylene glycol bis (β-amino-ethyl ether)-N, N'-tetraacetic acid; DOC, deoxycholate, SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

skeletal rabbit hind muscle was first isolated a decade ago by MacLennan (1). As a result, the enzyme from skeletal muscle has been characterized more extensively than the enzyme from cardiac muscle. In both systems, 2 moles of Ca^{2+} are transported for each mole of ATP hydrolyzed (11,12). The Ca^{2+} + Mg^{2+}-ATPase from skeletal and cardiac sarcoplasmic reticulum differ with respect to calcium binding, uptake, K_m for calcium and hydrolytic activity. They have similar K_m values for ATP (13). Recently two methods have been published for the isolation of the Ca^{2+} + Mg^{2+}-ATPase from cardiac sarcoplasmic reticulum (14,15). The method presented in this communication has the advantage over these two procedures in that it is a very rapid technique employing very low concentrations of detergent. Moreover, our method also isolates for the first time the 22,000 dalton protein from the cardiac sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURE

MATERIALS

Bovine heart cyclic AMP-dependent protein kinase, sodium cyclic AMP and Tris-ATP were purchased from Sigma Chemical Co. [γ-^{32}P] ATP, ammonium salt (20 mCi per mmol) was obtained from Amersham/Searle. Deoxycholate was purified by the method of MacLennan (1).

METHODS

Isolation of Cardiac Microsomes

Cardiac microsomes were prepared from canine heart ventricle according to Harigaya and Schwartz (16) with the following modifications. Cardiac ventricular muscle was homogenized in 10 mM $NaHCO_3$ and centrifuged at 8,700 x g for 20 min. The supernatant was then centrifuged at 10,000 x g for 20 min. The supernatant was strained through 4 layers of cheesecloth, followed by centrifugation at 37,000 x g for 30 min. The pellet was suspended in 20 mM Tris-maleate, 0.6 M KCl, pH 6.8, and centrifuged at 100,000 x g for 20 min. The pellet, containing microsomes, was suspended in 0.25 M sucrose.

Effect of Phosphorylation on the Solubility of the 22,000 Dalton Protein

When cardiac microsomes were phosphorylated first, and then subjected to treatment with DOC, the following procedure was employed. Cardiac microsomes (1.25 mg/ml) were suspended in 40 mM histidine buffer (pH 6.8),

0.12 M KCl, 0.5 mM $MgCl_2$, 2.5 mM EGTA, 5 mM NaN_3, 25 mM NaF, with 1 μM cyclic AMP and protein kinase (0.125 mg/ml). The reaction was started by adding 0.20 mM [γ - ^{32}P] ATP (1-3 μCi/nmol). The final volume was 1 ml. After 10 min, DOC was added at a concentration of 1.0 mg DOC/mg protein. The sample was incubated for an additional 10 min, followed by centrifugation at 100,000 x g for 30 min. Laemmli sample buffer (17) consisting of 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 3 mM LiCl, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue was added to both the pellet and supernatant. Both samples were separated on a 12% acrylamide - SDS - slab gel by the method of Laemmli and Favre (17). The gel was stained in 0.1% Coomassie blue, 50% methanol and 10% acetic acid, and diffusion destained in 10% acetic acid. The gel was dried on Whatman No. 5 filter paper. Varying concentrations of [γ - ^{32}P] ATP were also applied to the filter paper, so that the amount of ^{32}P incorporated into proteins could be quantitated. The dried slab gel was exposed to Kodak NS - 2T X-ray film, and the film was developed 5 days later. To quantitate the amount of ^{32}P incorporated into proteins, the film and gel were scanned with a Beckman densitometer.

When microsomes were first subjected to treatment by DOC and then phosphorylated, they were treated in the following manner. Cardiac microsomes were suspended in the phosphorylation buffer described above minus the cyclic AMP, protein kinase and ATP. DOC at concentrations from 0.1 mg DOC per mg protein to 1.0 mg DOC per mg protein was added. After 10 min of incubation, 1 μM cyclic AMP, protein kinase (0.125 mg/ml), and 0.20 mM [γ - ^{32}P] ATP were added. After an additional 10 min, the sample was centrifuged at 100,000 x g for 30 min. The pellet and supernatant were assayed for the presence of phosphorylated proteins as described above.

Disc Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis was run according to Weber and Osborn (18). Samples for electrophoresis were suspended in the SDS-sample buffer consisting of 1% SDS, 20 mM sodium phosphate (pH 7.2), 0.1 mM EDTA, 1% β-mercaptoethanol, 50% glycerol and 0.005% bromophenol blue. The samples were incubated at 37° for 1 hr. An aliquot containing between 50 and 150 µg was applied to the gel. After electrophoresis in the 10% polyacrylamide SDS-gel, the proteins were made visible by staining the gels in 0.25% Coomassie blue, 50% methanol and 10% acetic acid for 2 hr at 37°. The gels were diffusion destained in 10% acetic acid.

Phosphorylation Assay

Phosphorylation of cardiac microsomes, DOC supernatant and the purified 22,000 dalton protein was accomplished essentially by the method of Kirchberger et al. (19). The protein fraction (0.25 to 0.50 mg/ml) was suspended in 40 mM histidine (pH 6.8), 0.12 M KCl, 0.5 mM $MgCl_2$, 5 mM NaN_3, 25 mM NaF buffer. Cyclic AMP was added to a final concentration of 1 µM. Protein kinase was added at the concentration of 0.125 mg per ml. The reaction was started by the addition of 0.20 mM [γ - ^{32}P] ATP. The final volume was 0.2 ml. After 10 min, the reaction was stopped by adding 2 ml of cold 10% trichloroacetic acid, 0.1 mM KH_2PO_4. 0.2 ml of 0.63% bovine serum albumin was added as a carrier protein. After remaining on ice for 15 min, samples were centrifuged at 2,000 x g for 10 min. The pellets were suspended in 0.2 ml of 0.6 N NaOH. The protein was reprecipitated by the addition of 2 ml of 10% trichloroacetic acid, centrifuged, resuspended

in NaOH and washed a couple of times. The pellet was finally suspended in 0.2 ml of 0.6 N NaOH and transferred to scintillation vials containing 10 ml of Instagel (Packard) for counting.

ATPase Activity

Ca^{2+}-dependent ATPase was measured in a manner similar to that used by Tada et al. (3). Cardiac microsomes or the isolated protein were suspended at a concentration of 30-50 µg/ml in 40 mM histidine (pH 6.8), 0.125 M KCl, 5 mM NaN_3, 5 mM $MgCl_2$ and varying calcium concentrations in the presence of EGTA. The reaction was initiated by the addition of 5 mM [γ - ^{32}P] ATP (3 µCi per µmole). The final volume was 0.4 ml. The Ca^{2+}-independent ATPase activity was measured by including 0.5 mM EGTA instead of calcium-EGTA in the reaction mixture. At various time intervals, the reaction was stopped by adding 0.4 ml of ice-cold 13.33 mM silicotungstic acid in 1.67 M H_2SO_4. 0.3 ml of 10% ammonium molybdate was added. After vortexing, 1 ml of isobutanol:benzene (1:1) was added. The sample was vortexed for 30 sec, followed by centrifugation at 1,000 x g for 10 min. 0.5 ml of the organic phase was counted. To determine the nonspecific binding of phosphate, controls were run by adding the protein to the reaction mixture after the acid had been added. The free Ca^{2+} concentrations were determined by the method of Katz et al. (20).

Isolation of the 22,000 Dalton Protein

Cardiac microsomes were suspended at a concentration of 7 mg/ml in 0.25 M sucrose. A small aliquot of DOC was added at the ratio of 2 µg DOC/ 7 mg microsomal protein (0.286 µg DOC/mg protein). The suspension was

incubated on ice for 15 min. After incubation, the suspension was centrifuged at 100,000 x g for 30 min. The supernatant was concentrated on an Amicon PM 10 membrane. The supernatant was then applied to a Sephadex G-75 (fine) column (1.5 cm x 1.5 m), equilibrated with 10 mM Tris-HCl (pH 7.8), 0.04% DOC, 0.02% NaN_3. The sample was eluted from the column using the Tris-DOC buffer. The absorbance of the sample at 280 nm was monitored. The 22,000 dalton protein was eluted as a single protein as determined by SDS-polyacrylamide gel electrophoresis.

Isolation of the Ca^{2+} + Mg^{2+}-ATPase

As in the isolation of the 22,000 dalton protein, cardiac microsomes were suspended at 7 mg/ml in 0.25 M sucrose. DOC was added at the ratio of 2 µg DOC/7 mg protein. Each 7 mg of microsomal protein must be treated in a separate tube. The purification cannot be scaled upwards without losing about 10% in the purification of the ATPase. The mixture was incubated on ice of 15 min, followed by centrifugation at 100,000 x g for 30 min. The pellet was suspended in the original volume of 0.25 M sucrose, 1 ml. Additional DOC was added at the concentration of 2 µg DOC/7 mg of original microsomal protein (i.e. the same amount of DOC as was added initially). The suspension was incubated on ice for 15 min, followed by centrifugation at 100,000 x g for 30 min. The supernatant from this centrifugation contained the Ca^{2+} + Mg^{2+}-ATPase to at least 95% purity.

Biochemical Assays

Protein concentration was determined by the method of Lowry et al. (21) with bovine serum albumin as the standard. Phospholipid concentration was assayed by determining the phosphorus present by the method of Ames and Dubin (22).

RESULTS

Effect of Phosphorylation on the Solubility of the 22,000 Dalton Protein

When cardiac microsomes were phosphorylated and then treated with DOC, the phosphorylated 22,000 dalton protein was not soluble in DOC concentrations as high as 1.0 mg DOC/mg protein (Table I). As calculated from densitomer scans of the Coomassie blue - stained gels and the autoradiographs, the 22,000 dalton protein bound 0.40 moles phosphate/mole protein, when the microsomes were incubated in the presence of cyclic AMP and protein kinase for 10 min. The 22,000 dalton protein represents 5% of the total microsomal protein. Greater than 90% of the protein was not solubilized when phosphorylated prior to the addition of DOC.

Treating the microsomes first with DOC, followed by phosphorylation, resulted in 85% of the 22,000 dalton protein being solubilized by 0.1 mg DOC/mg protein, and subsequently phosphorylated. The presence of DOC did not affect the total amount of phosphorylation. Phosphorylation appears to alter the protein in such a manner as to render it inaccessible to DOC.

Purification of the 22,000 Dalton Protein

Since the unphosphorylated 22,000 dalton protein was soluble in a low concentration of DOC, this step was employed as the first step in the purification of the 22,000 dalton protein. DOC at a concentration of 2 µg DOC/7 mg protein rendered most of the 22,000 dalton protein soluble. After solubilization, a Sephadex G-75 column was used to purify the protein. The protein elution pattern from the column is shown in Fig. 1. SDS-polyacrylamide gels of the various column fractions are shown in Fig. 2. Gel A shows the proteins solubilized by the low concentration of DOC. These proteins included the 22,000 dalton protein, mainly higher molecular weight proteins and some of the ATPase. All solubilized proteins with a molecular weight above 60,000 were excluded in the column void volume, the major peak from the column (gel B). The first fraction separated by the column is shown in gel C. A 50,000 dalton protein was the major band with a few minor proteins also present. The isolated 22,000 dalton protein is seen in the next peak (gel D). The column was capable of separating the 22,000 dalton protein from a 18,000 dalton protein also solubilized by DOC. Gel E shows the next successive fractions. They contain mainly the 18,000 dalton protein with only a small amount of the 22,000 dalton protein eluting in this fraction.

The yield of the 22,000 dalton protein at each step of the purification is shown in Table II. The DOC solubilization step results in a yield of 0.37 mg of protein in the supernatant per 7 mg of sarcoplasmic reticulum. Usually about 2.7 mg of the solubilized proteins were applied to the Sephadex G-75 column. This results in a yield of about 150 µg of pure 22,000 dalton

protein. About 100 µg of pure 22,000 dalton protein can be obtained from 34 mg of sarcoplasmic reticulum.

Phosphorylation of the Purified 22,000 Dalton Protein

The isolated 22,000 dalton protein was specifically phosphorylated by cyclic AMP-dependent protein kinase as is shown in Table III. Cardiac microsomes incorporated approximately 1.35 nmoles ^{32}P per mg protein. This data is consistent with that reported by Kirchberger et al. (19). The supernatant from the first DOC solubilization incorporated only about a quarter of the phosphate that the microsomal fraction did. The isolated 22,000 dalton protein incorporated 6.83 nmoles phosphate per mg protein, or 0.15 moles of phosphate per mole of protein.

Phospholipid Content of the 22,000 Dalton Protein

Phospholipid content during the purification steps is detailed in Table IV. Cardiac microsomes contained 20 µg phospholipid phosphorus/mg protein. The solubilized protein fraction contained only 5 µg phospholipid phosphorus/mg protein. The purified 22,000 dalton protein bound 5 moles of phospholipid/mole protein. The fact that phospholipid is tightly associated with the 22,000 dalton protein, is consistent with the finding that the 22,000 dalton protein can be solubilized by acidified chloroform:methanol (4).

Isolation of the Ca^{2+} + Mg^{2+}-ATPase

The purification of the cardiac Ca^{2+} + Mg^{2+}-ATPase was accomplished by selective solubilization of the membrane proteins by DOC. Figure 3 shows the gel pattern of the purification procedure. The Ca^{2+} + Mg^{2+}-ATPase represents about 30% of the total protein present in cardiac sarcoplasmic reticulum (gel A). The first treatment with DOC selectively solubilized the extrinsic proteins including the 22,000 dalton protein and some of the ATPase (gel B). Adding an additional small amount of DOC to the pellet from the first extraction resulted in the isolation of the ATPase to at least 95% purity, as shown in gel C. This procedure yields 50 ± 5 µg of purified Ca^{2+} + Mg^{2+}-ATPase per mg of sarcoplasmic reticulum. The isolated enzyme contained no phosphorylase activity (Data not shown). The purified Ca^{2+} + Mg^{2+}-ATPase bound 11.5 moles of phospholipid/mole protein (Table IV).

IG. 3

ATPase Activity

Cardiac microsomes hydrolyzed 0.42 µmoles ATP/mg protein /min (Table V). About 40% of the hydrolytic activity was Ca^{2+}-independent. Once the ATPase was solubilized virtually all the hydrolytic activity was Ca^{2+}-dependent. Even in the supernatant from the first solubilization with DOC, all the ATPase activity was Ca^{2+}-dependent. The purified enzyme exhibited activity in the range of 1.2 µmoles Pi generated/mg protein/min. By adding additional DOC to the purified enzyme, the hydrolytic activity doubled. DOC was also found to stimulate the ATPase activity of the Ca^{2+} + Mg^{2+}-ATPase isolated from skeletal muscle (1). The K_m for the purified Ca^{2+} + Mg^{2+}-ATPase from cardiac muscle was found to be 5.2 µM Ca^{2+}. This value is in agreement with the value obtained from cardiac microsomes (13).

ABLE V

DISCUSSION

Selective solubilization of membrane proteins by detergents is a powerful tool in protein purification. The 22,000 dalton protein, specifically phosphorylated by cyclic AMP-dependent protein kinase, has a very unique property. When phosphorylated, it cannot be solubilized by DOC. However, unphosphorylated, the protein is soluble in a low concentration of detergent. Phosphorylation of the 22,000 dalton protein appears to cause the protein to become buried in the membrane, rendering it resistant to DOC and trypsin (3,4). How the addition of a phosphate group to the protein can significantly change the properties of the protein is intriguing, but not understood. The fact that the nonphosphorylated 22,000 dalton protein was solubilized by a low concentration of DOC, was used in the purification of the protein. After selective solubilization, only a Sephadex G-75 column was needed to completely purify the protein. The purified protein was specifically phosphorylated by cyclic AMP-dependent protein kinase.

The isolated 22,000 dalton protein bound 6.83 nmoles of phosphate per mg of protein, compared to the sarcoplasmic reticulum, which bound 1.35 nmoles of phosphate per mg protein. This is not as great an enhancement as might be anticipated, considering that the 22,000 dalton protein accounts for only 5% of the total microsomal protein. However, in the sarcoplasmic reticulum, more than one protein is phosphorylated. The 22,000 and 6,000 dalton proteins are the major ones, along with the endogenous protein kinase and phosphorylase a. As determined by slab gel electrophoresis and autoradiography, the 22,000 dalton protein bound

0.40 moles phosphate/mole protein, when cardiac microsomes were incubated for 10 min in the presence of cyclic AMP and protein kinase. The isolated protein bound 0.15 moles of phosphate per mole of protein. This degree of incorporation is considerably lower than the idealistic, 1 mole phosphate per mole of protein, but is in agreement with the finding for other isolated proteins. Specific cyclic AMP-dependent protein kinase phosphorylation of isolated ribosomal proteins has shown that between 0.035-0.069 moles of phosphate were incorporated per mole of protein (23,24). Glycogen synthetase (25) has been shown to incorporate approximately 1 mole of phosphate per mole of protein, while phosphorylase kinase incorporates 0.4 moles of phosphate per mole of protein (26). Thus, there seems to be a large range of values for the specific incorporation of a phosphate into a protein by cyclic AMP-dependent protein kinase. The purified 22,000 dalton protein appears to bind only about 40% of the phosphate that the protein did in the microsomal fraction. This may be the case, or it may be the result of error introduced by the densitomer scans of the Coomassie blue stained gels and autoradiographs. Whether the Coomassie blue stained gels give an accurate report of the amount of phospholamban present in the microsomes is not known. Regardless, the purified 22,000 dalton protein is specifically phosphorylated by cyclic AMP-dependent protein kinase at a high enough specific activity that the function of the phosphorylated 22,000 dalton protein can be elucidated.

When isolated, the 22,000 dalton protein still has phospholipids bound to it. This fact is consistent with the finding that the 22,000 dalton protein is soluble in acidified chloroform:methanol, indicating the

possibility that it is a proteolipid (4). Whether these lipids play any direct role in the function of the 22,000 dalton protein is not known. It has been shown that the protein, not the lipid, is the component phosphorylated by cyclic AMP-dependent protein kinase (3,4).

The Ca^{2+} + Mg^{2+}-ATPase was isolated by employing successive additions of DOC. This procedure allowed for the very rapid purification of the ATPase. Because of solubilizing the enzyme in a very low concentration of detergent, fewer phospholipids were associated with the enzyme than found in the purified ATPase from skeletal muscle isolated by MacLennan (1). However, the phospholipid content of the isolated ATPase from cardiac muscle is in agreement with the phospholipid content associated with the enzyme purified from skeletal muscle by Warren et al. (27). They also employed a low concentration of detergent. With the isolated enzyme from skeletal muscle, MacLennan (1) found a greater hydrolytic activity than Warren et al. (27). The amount of phospholipid associated with the enzyme undoubtedly affects the hydrolytic activity of the enzyme. The increased activity seen in the presence of added DOC, may be a result of DOC stabilizing the enzyme in a manner similar to that of phospholipids. The K_m value for calcium found for the purified cardiac enzyme is in agreement with the value reported for cardiac microsomes, and is about 5 times greater than the value for the ATPase from skeletal muscle (13,14).

Using the procedures presented here, the rapid purification of the 22,000 dalton protein and the Ca^{2+} + Mg^{2+}-ATPase is possible. A good yield of the 22,000 dalton protein (approximately 200 µg) can be obtained

from as few as 2 canine hearts, while the ATPase can be isolated from 1 heart. With the isolation of both these proteins, it will now be possible to elucidate the function of cyclic AMP-dependent phosphorylation of the 22,000 dalton protein in the regulation of calcium transport.

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FIGURE LEGENDS

Figure 1: Elution profile from the Sephadex G-75 column. Cardiac microsomes were suspended at 7 mg/ml in 0.25 M sucrose. 2 µl of 0.1% DOC was added per 7 mg protein. The microsomes were centrifuged at 100,000 x g for 30 min. The supernatants were concentrated and 2.7 mg of protein was applied to a Sephadex G-75 (fine) column (1.5 cm x 1.5 m) equilibrated with 10 mM Tris (pH 7.8), 0.04% DOC, 0.02% NaN_3. The absorbance of the eluant was monitored at 280 nm. Each fraction was 4.7 ml.

Figure 2: Coomassie blue stained gels of the column fractions. 10% Weber and Osborn polyacrylamide-SDS-gels were run using the column fractions shown in Fig. 1. The gel on the far left (A), shows the DOC solubilized supernatant that was applied to the column. Gel B shows the proteins in the void volume, fractions 25-30. Gel C shows fractions 31-38. Gel D shows the purified 22,000 dalton protein, fractions 39-51. Gel E shows the fractions 52-61, showing the 18,000 dalton protein with a small amount of the 22,000 dalton protein present.

Figure 3: Coomassie blue stained 10% Weber and Osborn polyacrylamide-SDS-gels of the isolation of the Ca^{2+} + Mg^{2+}-ATPase. Gel A shows the cardiac microsomes. Gel B shows the first supernatant after treating the microsomes with DOC. Gel C shows the purified ATPase. The band above the 100,000 dalton band represented a dimer of the ATPase.

TABLE I

The Effect of Phosphorylation
on the Susceptibility of the 22,000 Dalton Protein
to Solubilization by DOC

	moles of phosphate/mole of 22,000 dalton protein	
	pellet	supernatant
Phosphorylation followed by:		
Control	0.39 ± 0.04	0.01 ± 0.01
DOC (1.0 mg/mg protein)	0.37 ± 0.03	0.02 ± 0.01
DOC (0.1 mg/mg protein) followed by:		
Cyclic AMP + Protein Kinase	0.04 ± 0.01	0.36 ± 0.05

In the top half of the table, microsomes were phosphorylated for 10 min as described in "Methods". After 10 min, 1.0 mg DOC/mg protein was added, and the sample was centrifuged at 100,000 x g for 30 min. Laemmli solubilizing buffer was added to the pellet and supernatant and 50 µg of each was electrophoresed on a 12% acrylamide-SDS-slab gel. After autoradiography, the percent of ^{32}P label in each band was determined by scanning the autoradiograph with a Beckman densitometer. DOC was not added to the control. In the bottom half of the table, DOC was added first to the microsomes. After 10 min, cyclic AMP, protein kinase and [γ-^{32}P] ATP were added. After an additional 10 min, the samples were centrifuged at 100,000 x g for 30 min and assayed as described above. The data represent the mean ± S.E. for 6 experiments.

TABLE II

Percent Protein Obtained in Each Isolation Step

Isolation Step	Protein Yield, %	22,000 Dalton Protein, %
Cardiac microsomes	100.0	5.1
DOC supernatant	5.3	10.8
Isolated protein	0.3	100.0

Percent protein yield during isolation of the 22,000 dalton protein. The percent protein at each step of the purification was calculated by taking the cardiac microsomes as 100%. The percent of 22,000 dalton protein at each step was determined by scanning Coomassie blue stained 10% Weber and Osborn polyacrylamide-SDS-gels at 550 nm.

TABLE III

Cyclic AMP-dependent Phosphorylation

Protein Fraction	nmoles ^{32}P/mg protein	moles ^{32}P/mole protein
Cardiac microsomes	1.35 ± 0.16	
First DOC supernatant	0.31 ± 0.08	
Purified 22,000	6.83 ± 0.31	0.15 ± 0.02

Cyclic AMP-dependent phosphorylation of the sarcoplasmic reticulum, DOC supernatant and the purified 22,000 dalton protein. Each fraction was incubated in the phosphorylating buffer, including [γ - ^{32}P] ATP in a final volume of 0.2 ml. After 10 min, the phosphorylation was stopped by the addition of 2 ml of 10% trichloroacetic acid (TCA), 0.1 mM KH_2PO_4. 0.2 ml of 0.63% bovine serum albumin was added as a carrier protein. After incubating on ice for 15 min, the sample was centrifuged at 2,000 x g for 10 min. The pellet was solubilized by 0.6 N NaOH, reprecipitated with 10% TCA, 0.1 mM KH_2PO_4 and washed two times. Finally, the pellet was solubilized in NaOH, transferred to 10 ml Instagel (Packard) and the radioactivity was counted. The data represent the mean ± S.E. for five experiments on the cardiac microsomes, and three determinations on two different preparations for the DOC supernatant and the purified 22,000 dalton protein.

TABLE IV

Phospholipid Content

	Lipid Content	
	µg phospholipid phosphorus/mg protein	mole phospholipid phosphorus/mole protein
Cardiac microsomes	20.5 ± 0.8	
First DOC supernatant	5.1 ± 0.4	
22,000 dalton protein	7.2 ± 0.5	5.1 ± 0.02
Ca^{2+} + Mg^{2+}-ATPase	3.6 ± 0.4	11.5 ± 0.03

Phospholipid content of the sarcoplasmic reticulum, DOC supernatant, 22,000 dalton protein, and Ca^{2+} + Mg^{2+}-ATPase. The phosphorus present in each sample was determined by the method of Ames and Dubin (22). The experimental results are presented as the mean ± S.E. for five experiments.

TABLE V

ATPase Activity

Protein Fraction	Mg^{2+}-ATPase	Ca^{2+}-dependent ATPase
	µmoles Pi/mg/min	µmoles Pi/mg/min
Cardiac microsomes	0.18 ± 0.03	0.24 ± 0.06
First DOC supernatant	0.02 ± 0.03	0.73 ± 0.10
Purified Ca^{2+} + Mg^{2+}-ATPase	0.01 ± 0.02	1.27 ± 0.15
Purified Ca^{2+} + Mg^{2+}-ATPase + 0.1% DOC	0.01 ± 0.03	2.47 ± 0.31

The ATPase hydrolytic activity of cardiac microsomes, DOC supernatant and purified Ca^{2+} + Mg^{2+}-ATPase. The hydrolytic activity was determined as described in "Methods". The Ca^{2+}-independent ATPase activity was measured by including 0.5 mM EGTA instead of calcium-EGTA in the reaction mixture. The data represents the mean ± S.E. for five experiments.

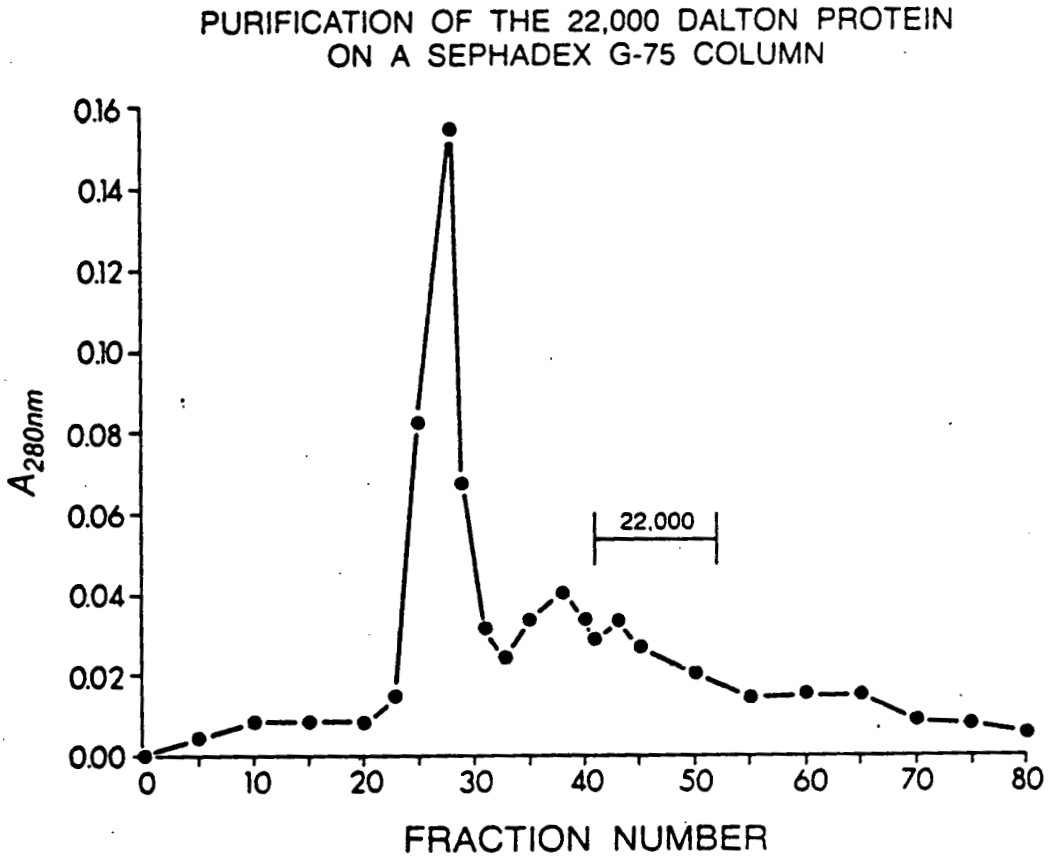
PURIFICATION OF THE 22,000 DALTON PROTEIN
ON A SEPHADEX G-75 COLUMN
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A_{280nm}
22,000
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 1

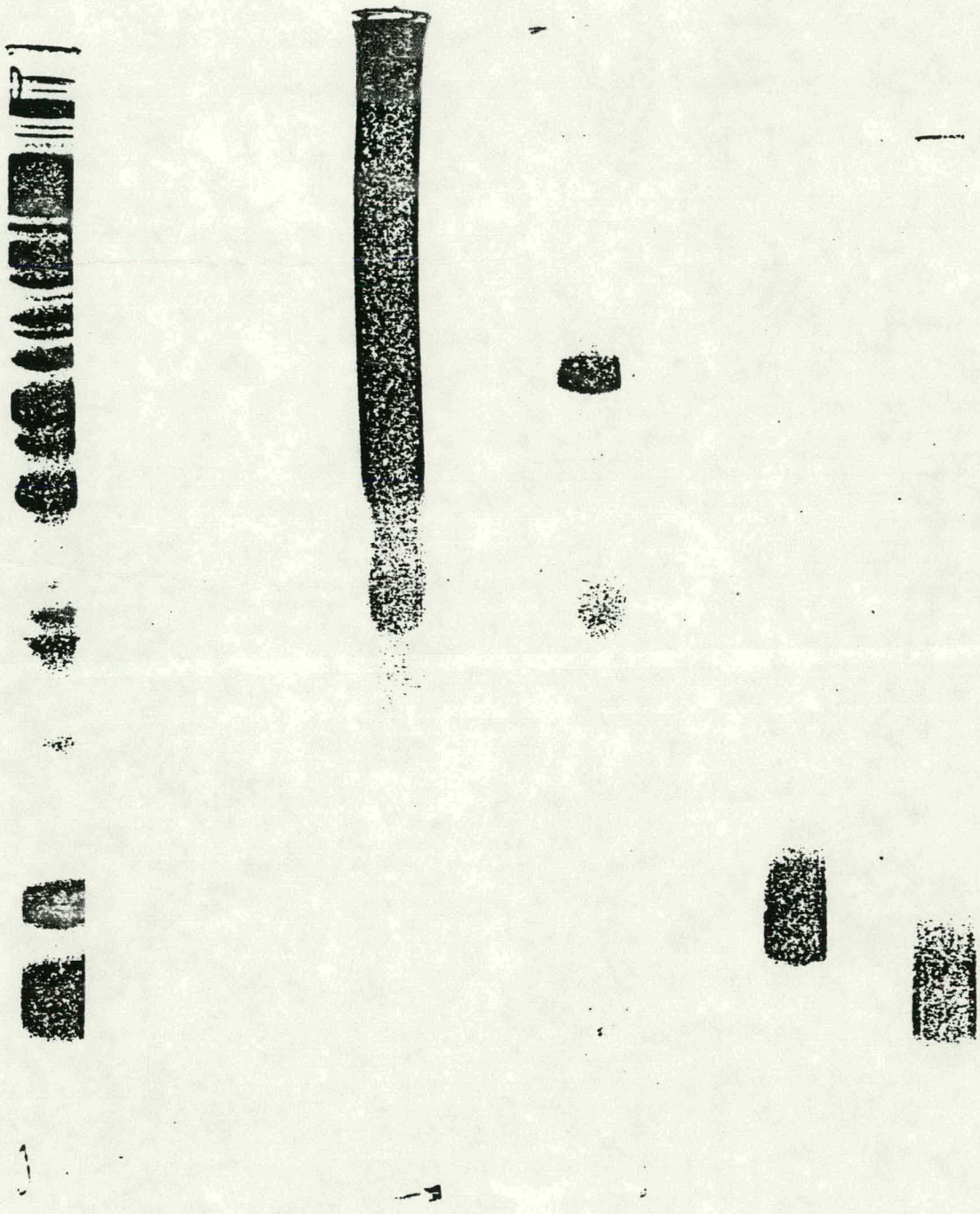

Fig. 2

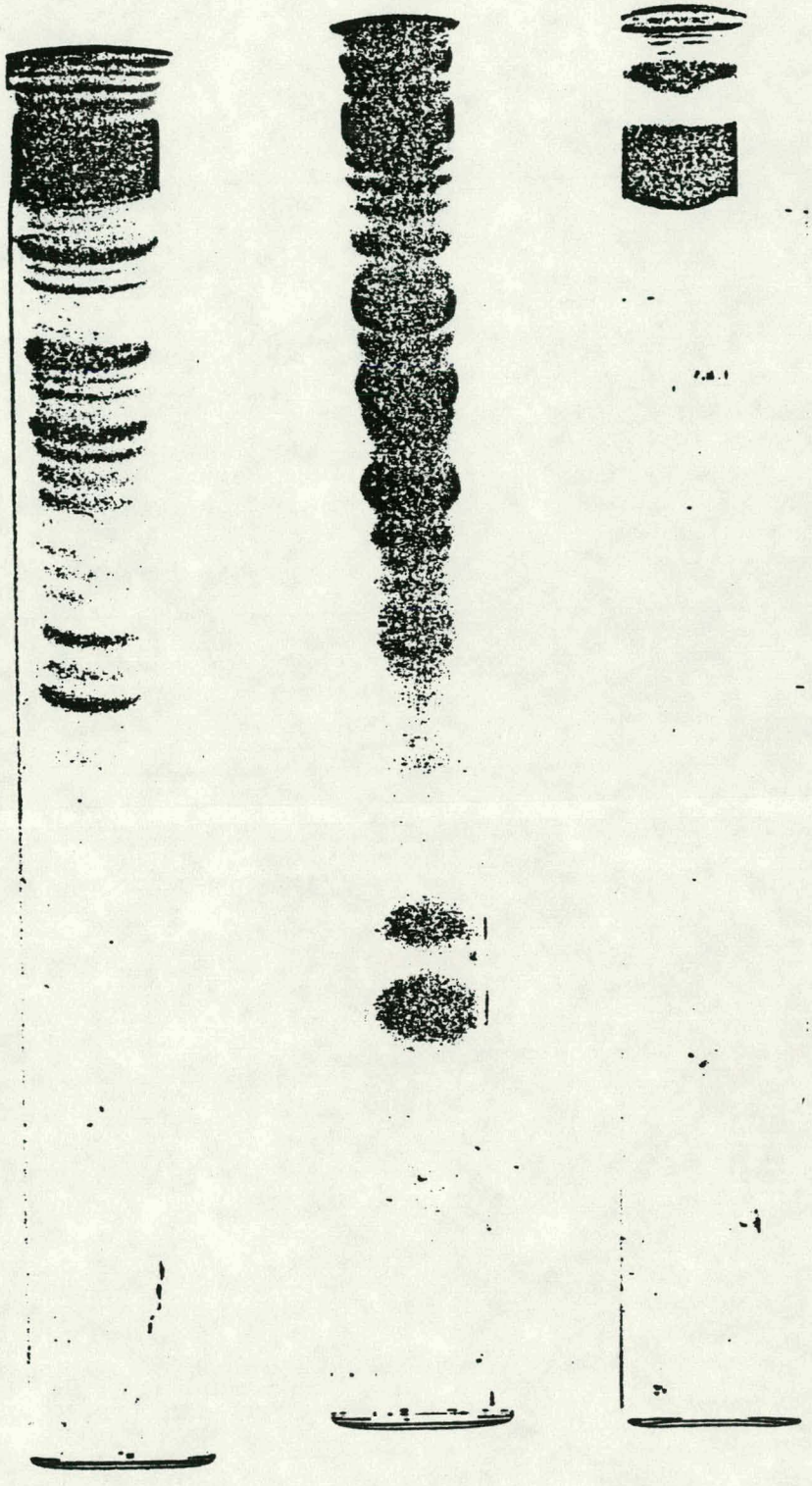

Fig. 3